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Short communication

Biohydrogen production from palm oil mill effluent using immobilized *Clostridium butyricum* EB6 in polyethylene glycol

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ABSTRACT

A novel polyethylene glycol (PEG) gel was fabricated and used as a carrier to immobilize *Clostridium butyricum* EB6 to improve biohydrogen (bio-H_2) production from palm oil mill effluent (POME). POME is used as a substrate that can act as a carbon source. The resulting PEG-immobilized cells were found to yield 5.35 LH_2/L-POME, and the maximum H_2 production rate was 510 mL H_2/L-POME h (22.7 mmol/L h). The Monod-type kinetic model was used to describe the effect of substrate (POME) concentration on the H_2 production rate. The acclimation of immobilized cells greatly improved H_2 production. Batch experiments demonstrated that particle size of PEG-immobilized cells for efficient H_2 production 3 mm. It is significant that this is the first report on whole-cell immobilization in PEG for H_2 production from POME.

1. Introduction

A major predicted worldwide trend is the shift away from non-renewable fossil fuels toward the establishment of renewable H_2 energy. Unlike fossil fuels, H_2 is a promising clean fuel with zero carbon emission at the point of use and a high energy yield of 142.35 kJ/g, which is 2.75 times that of any hydrocarbon fuel [1]. Current H_2 production technology can be classified as chemical–physical or biological. Chemical–physical methods (e.g., fossil fuel processing or electrolysis) are highly energy intensive and result in the emission of harmful greenhouse gases that impact on global warming [2]. In contrast, biological methods for H_2 production are environment friendly, lower in energy consumption and cheaper because H_2 can be produced from raw materials such as organic wastes under mild operating conditions [3]. In recent years, anaerobic H_2 fermentation from wastewater and solid biomass has received considerable attention. Much research has been conducted on the generation of H_2 from actual wastewater [4–7]. In Malaysia, the estimated annual production of palm oil mill effluent (POME) is about 50 million tons. POME is a colloidal suspension consisting of 92–94% water and 6–7% total solids (TS) with a high chemical oxygen demand (COD) of 75–86 g/L and a biochemical oxygen demand (BOD) of 23–55 g/L. The potential of using POME as a fermentation medium to produce H_2 gas has been recently researched [8–11]. Most studies on H_2 production have used free cell systems. These systems are usually ineffective or difficult to handle in continuous operation, and the recycling of biomass is considered necessary to maintain sufficient cell concentration in the reactor to maximize H_2 production [12].

Cell-immobilization technology provides an alternative approach to suspended-cell systems and improves H_2 production. Immobilized-cell systems are effective in enhancing biomass retention and can operate at higher dilution rates without biomass washout from the reactor [13]. Such systems produce almost cell-free effluent that is more suited to energy recovery from waste or wastewater [14,15]. Much research has been conducted on cell immobilization by natural and synthetic polymers for batch and continuous bio-H_2 production from synthetic wastewater [16–18]. However, no information is available in the literature regarding the use of cell immobilization technique for H_2 production from real wastewater (such as POME). In this work, a new material for cell immobilization, namely polyethylene glycol (PEG), was employed to entrap the *Clostridium butyricum* EB6, and its H_2 production performance was investigated. PEG prepolymer was selected as the solid matrix for its low toxicity, simple immobilization procedure, low cost and highly porous structure that helps to sustain

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immobilized cell viability [19]. Batch fermentation was carried out to explore the effect on H_2 production of substrate concentration (POME, expressed as COD) and the size of immobilized beads.

2. Materials and methods

2.1. Substrate and inoculum

Raw POME was collected from the tank of the palm oil mill at Kilang Sawit Lepar Hilir Pahang and was used as the substrate (carbon source) for H_2 fermentation. The POME was preserved at 4 °C to prevent self-biodegradation and acidification. The characteristics of POME used in this study are summarized in Table 1. The strain for hydrogen producing *C. butyricum* EB6 was used as inoculum in this study. *C. butyricum* EB6 was isolated from POME sludge and it was proved that this strain is capable of producing hydrogen via anaerobic fermentation [10]. The culture medium of strain consisted of (per liter) 10 g of meat extract, 0.5 g of starch, 4 g of glucose, 4 g of peptone, 3 g of yeast extract, 0.5 g of agar, 0.5 g of L-cysteine·HCl·H_2O, 5 g of NaCl and 2 g of CH_3COONa. The bacterium was stored in 10% glycerol at −10 °C before being subjected to immobilization.

2.2. Cell immobilization

C. butyricum EB6 cells were immobilized by entrapment into PEG prepolymer. First, 10% (w/v) PEG and 0.6% (w/v) N,N′-methylenebisacrylamide (MBA) crosslinker were dissolved in a water. The resulting mixture and 20 ml of inoculum (in exponential growth phase) was quickly mixed in a beaker. The dry weight of cells in 20 ml of inoculum was approximately 2.2 g. To start polymerization, 0.25% (w/v) potassium persulfate initiator ($K_2S_2O_8$) was mixed in and the mixture allowed to stand for about 20 min to promote bead formation. The resulting immobilized cell bead was cut into 3-mm beads (density 1.42 g/cm^3). The biomass content of the immobilized bead was ~10 mg cell/g bead.

2.3. Batch operation for H_2 production

A total of 300 g of immobilized beads was inoculated in a 2-L bioreactor with 1L raw POME as a substrate. Anaerobic conditions were created in the bioreactor by nitrogen gas sparging for 10 min. The batch tests were performed to determine the effects of different sizes of immobilized beads on H_2 production. The bioreactor was maintained at a constant temperature of 37 °C and a pH of 5.5, which were found to be favorable for H_2 production [10]. The bioreactor was capped with a stopper and placed in a reciprocal shaker (150 rpm). At the end of each experiment, the beads collected from the spent medium were washed with distilled water. As a control, the test was also conducted with free cells (without immobilization) to examine the H_2 production. All tests were conducted in triplicate.

2.4. Analytical methods

A gas chromatograph (GC 8500 Perkin Elmer) equipped with a thermal conductivity detector and a 2-m stainless-steel SS350A column packed with a molecular sieve (80/100 mesh) was used to determined the fraction of H_2 in biogas using nitrogen as a carrier gas at a flow rate of 25 mL/min. The operating temperatures of the injection port, oven and detector were 100 °C, 85 °C and 100 °C, respectively. Methane and carbon dioxide were analyzed using the same model GC with a 2-m stainless-steel column packed with Porapak T (60/80 mesh) using helium as a carrier gas at a flow rate of 30 mL/min. The operating temperatures of the injection port, oven and detector were 150 °C, 80 °C and 100 °C, respectively [20]. The gas sample of 50 μl for methane and 250 μl for H_2 was injected in duplicate. VFA contents of filtered sample (0.2 mm) were determined by GC with a flame ionization detector (model 6890N, Agilent Inc., glass 2m × 2 mm packed column Carbopack B-DA 80/120% CW 20 M, N_2 carrier at kPa). The temperature of the injector port was 250 °C. The chromatography was performed using the following program: 100 °C for 5 min, 100–250 °C with a ramping of 10 °C/min, 250 °C for 15 min. The detector temperature was set 300 °C. For alcohol analysis, 1 cm^3 of sample acidified with 0.003 cm^3 25% H_2SO_4, was analyzed using GC–FID and capillary column of the same model and type, respectively. Alkalinity was determined by direct titration method. Measurements of COD, BOD, TS and VSS were carried out using standard methods [21]. The dry weight of immobilized cell in immobilized beads was assessed by measuring the difference in dry weight between the biomass-associated beads and the beads alone [20].

Table 1
Characteristics of palm oil mill effluent.

Parameter	Concentration (mg/L)
Biochemical oxygen demand (BOD)	23,100–55,200
Chemical oxygen demand (COD)	55,100–86,300
pH	4.0–5.0
Total carbohydrate	16,200–20,000
Total nitrogen	820–910
Ammonium -nitrogen	25–35
Total phosphorus	95–120
Phosphorus	14–20
oil	2000–2500
Total solid (TS)	30,000–42,000
Volatile suspended solids (VSS)	8400–12,000
Alkalinity	100–150

All values are in mg/L except pH.

Table 2
Comparative study on the efficiency of hydrogen fermentation processes.

Fermentation process	Substrate	Types	H_2 production rate	References
Batch	Sugarcane juice	IMC	5.78 mmol/L h	[18]
Continuous	Glucose	IMC	2.76 mmol/L h	[16]
Batch	POME	IMC	22.7 mmol/L h	Present work
Batch	POME	SPC	12.3 mmol/L h	Present work
Continuous	POME	SPC	16.9 mmol/L h	[8]
Feed batch	POME	SPC	19.4 mmol/L h	[9]
Continuous	POME	SPC	12.4 mmol/L h	[11]
Batch	Food wastewater	SPC	97.5 mmol/L day	[23]

IMC: immobilized cells system.
SPC: suspended cells system.

2.5. Scanning electron microscopy analysis

The immobilized cells were washed with distilled water and treated with 2% glutaraldehyde for 2 h. The samples were dehydrated by graded ethanol solutions (50–90%) for 20 min in each solution. The dehydrated immobilized cells were transferred into a freeze dryer. The dried samples were covered with a layer of gold under vacuum prior to being subjected to SEM (Zeiss EVO50, Germany).

3. Results and discussion

3.1. Performance of immobilized cells compared to that of free cell cultures

A comparison of a H_2 production rate by free and immobilized cells of *C. butyricum* EB6 was done by running batch fermentations test. In this H_2 production rate values obtained from the PEG entrapment technique were greater than those obtained from the free cells experiments (Table 2). The results suggested that the immobilization technique could improve the hydrogen production efficiency of *C. butyricum* EB6 from real wastewater. This could be due to the fact that the cell wall membrane system interacts strongly with the gel matrix network. Since this network can present different structural arrangements, unconventional three-dimensional adjustments between the cell wall and the matrix molecules are possible. Therefore, these altered spatial organizations at discrete molecular levels induce the cells at determined specific conditions to enhance the biosynthesis of metabolites and increase the permeability of the cells inside the bead to the substrate [22], hence enhancing the rate of H_2 hydrogen production. Fig. 1 shows the effect of time on the cell leakage from the beads in the medium suspension and consequent effect on hydrogen production. The prolongation of experiment time from 4 h to 24 h the cell leakage in the suspension medium is negligible, which resulted in slightly increase in hydrogen production rate. The SEM photographs show that structure of PEG beads had lot of pores inside the gels, which was a suitable for the entrapment of microbial cells and provided an anaerobic environment for the growth of the microorganisms (Fig. 2).

Table 2 lists the H_2 production rates in the literature with free and immobilized cells from different substrate for comparison. Although the H_2 production obtained from the present study is still lower than that obtained from food wastewater using free cells culture [23]. The possible reasons for higher H_2 production from

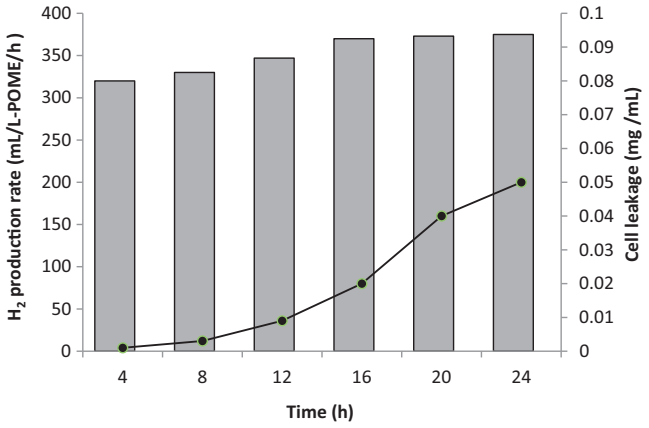

Fig. 1. The effect of time on the cell leakage from the beads in the medium suspension and consequent effect on hydrogen production.

food wastewater could be due to the different substrate, their COD concentration ranges and reaction conditions.

3.2. Effect of acclimated immobilized cells on H_2 production

In the current study, immobilized cells tended to have enhanced H_2 production rates when they were repeatedly adapted with POME medium for a period of time. Therefore, to increase H_2 production rates, the immobilized cells were repeatedly cultivated in POME until an optimal H_2 production rate was obtained. Significant improvement in H_2 production rate was observed with acclimated immobilized cells (see Fig. 3) compared with unacclimated immobilized cells. The lag phase of H_2 production by acclimated immobilized culture was in the range 1–3 h, which was lower than the lag phase of 7–12 h for unacclimated immobilized culture. As shown in Table 3, there were significant differences in H_2 production rates (R_{H_2}) and yields between the immobilized culture with and without acclimation. Acclimated immobilized culture gave a 1.5–5.0-fold improvement in R_{H_2} and 1.5–2-fold increases in yield compared with unacclimated immobilized cells. Enhanced H_2 production activity of immobilized cells with acclimation could be due to the exposure of cells to the substrate may trigger the production of the necessary enzymes involved in the utilization of the substrate more efficiently for H_2 production.

A maximum H_2 production rate of 510 mL H_2/L-POME h was attained when the acclimated immobilized cells were cultivated at an initial substrate concentration of 60,000 mg COD/L-POME. In response to changes in initial substrate concentration (20,000–60,000 mg COD/L-POME), the H_2 yield was slightly increased from 5.11 to 5.35 LH_2/L-POME (Table 3). It is likely that the change in organic loading of 20,000–60,000 mg COD/L-POME did not affect the microbial community structure in the immobilized cells. This work revealed that acclimation plays a crucial role in speeding up H_2 production from real wastewater (POME).

3.3. Kinetics of H_2 production with immobilized cultures using POME substrate

The dependence of the R_{H_2} of immobilized cells (with and without acclimation) on different POME concentrations is shown in Fig. 4. The R_{H_2} increased initially with increase in POME

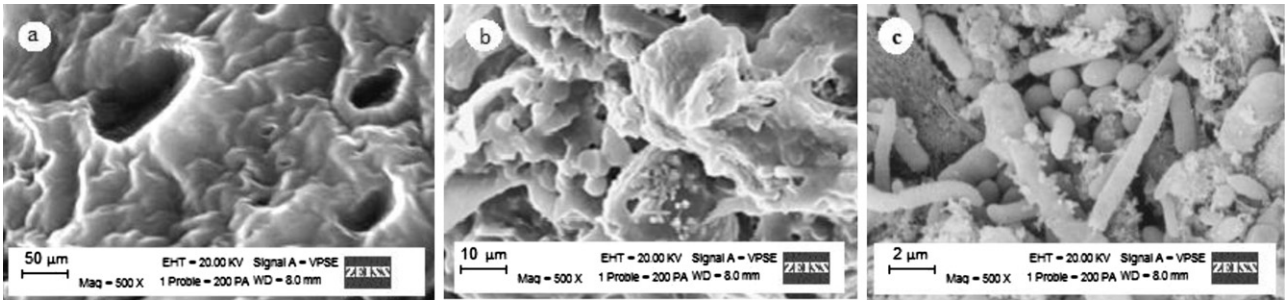

Fig. 2. SEM images of the PEG-immobilized cells. (a) Immobilized beads without cells. Scale bar: 50 μm. (b) Immobilized beads with cells. Scale bar: 10 μm (c) Cross-sectional image of the immobilized beads with cells. Scale bar: 2 μm.

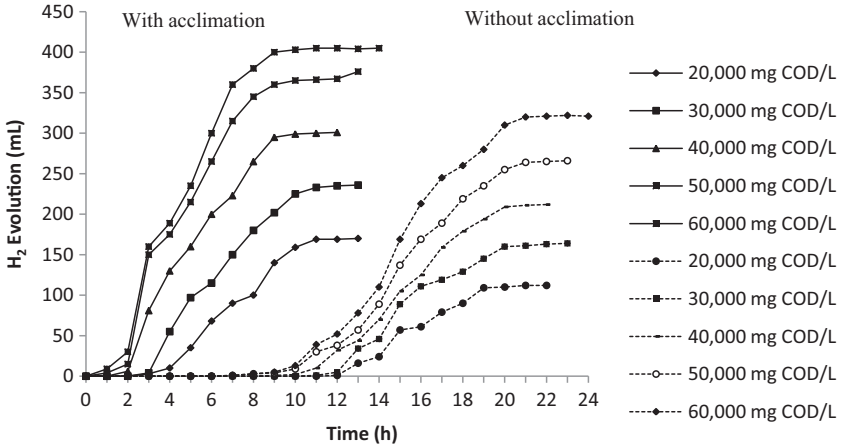

Fig. 3. The effect of acclimation on H_2 production performance for PEG-immobilized cells; the initial POME concentration = 40,000 mg COD/L; T = 37 °C; pH = 5.5; biomass loading = 30 g of cell/L; (black line: result from immobilized acclimated cells; dotted line: result from without acclimation).

Table 3
Effect of different POME concentrations on H_2 production performance with PEG immobilized cells.

	Substrate concentration (mg COD/L-POME)	H_2 production rate (mL H_2/L-POME h)	H_2 yield (LH_2/L-POME)	H_2 content (%)	H_2 evolution (mL)
Without acclimation	20,000	92	3.14	50.1	112
	30,000	144	3.55	50.3	164
	40,000	268	3.51	51.5	212
	50,000	310	3.81	51.2	266
	60,000	349	3.91	52.1	321
With acclimation	20,000	168	5.18	57.1	170
	30,000	315	5.22	60.0	236
	40,000	481	5.35	58.8	301
	50,000	461	5.11	60.0	376
	60,000	510	5.19	59.9	403

concentration and then leveled off at a maximum value as POME concentration exceeded 30,000–50,000 mg COD/L-POME. The relation between the medium concentration (POME) and R_{H_2} an be explained by Monod-type kinetics:

$$R_{H_2} = \frac{R_{max,H_2} S_{POME}}{K_P + S_{POME}} \quad (1)$$

where K_P is the half-saturation constant (mg COD/L), R_{max,H_2} is the maximum specific H_2 production rate (mL/g of cell/h) and S_{POME} is the POME concentration (mg COD/L). The correlation r^2 value obtained by the nonlinear regression analysis of Eq. (1) was greater than 0.92 for immobilized cells. The values of K_P and R_{max,H_2} were estimated from the experimental data by numerical simulation. For acclimated immobilized cells, the K_P and R_{max,H_2} values were 20,000 mg COD/L and 490 mL/g of cell/h, respectively, whereas for unacclimated cells, the values were 30,000 mg COD/L and 315 mL/g of cell/h, respectively. Fig. 3 shows that acclimated immobilized cells had significantly higher R_{max,H_2} values than those obtained from unacclimated cells. R_{max,H_2} value in the Monod model is considered a liner function of total active biocatalyst concentration. Therefore, the higher R_{max,H_2} value for acclimated immobilized cells represents higher concentration of active biocatalyst than that of unacclimated immobilized cells.

3.4. Effects of immobilized bead size on H_2 production

The effect of three different PEG bead sizes (3, 4 and 5 mm) on the H_2 production rate was assessed while keeping the PEG bead dosage (300 g) constant. As shown in Fig. 5, the maximum rate of 427 mL H_2/L-POME h was observed with bead size of 3 mm. However, the lower hydrogen production rate was observed when the bead size was at 4 and 5 mm. The differences in H_2 production performance may result from the cells immobilized in PEG with

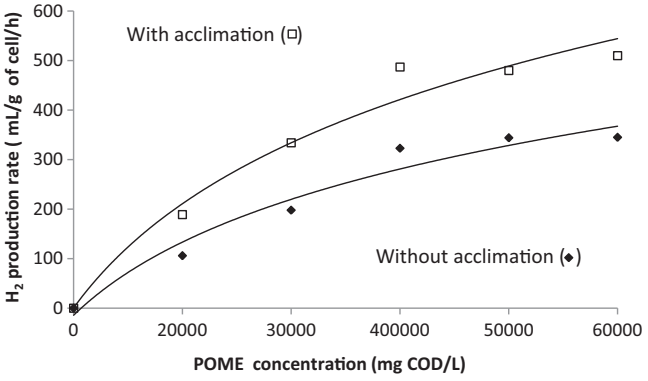

Fig. 4. The dependence of H_2 production rate on different POME concentration for PEG-immobilized cells. The initial biomass loading was 30 g of cell/L; T = 37 °C; pH = 5.5.

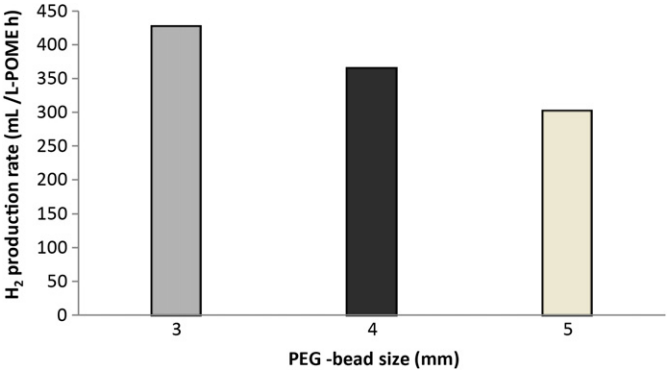

Fig. 5. Effect of PEG beads size on H_2 production rate by immobilized cells.

different bead size. In previous work has confirmed that fluidization quality affected by particle size [24]. At the same velocity, different particle size groups show different flow patterns. The particle size is the factor that differentiates the particle motion inside the reactor [25]. In this study, the bead size (3 mm) was observed fluidized and evenly distributed in the medium during the stirrer operation at 150 rpm, so the cells immobilized in the carrier to provide better distribution of biomass and more active exchange of cells with the wastewater. Therefore, more H_2 production was attained by bacteria immobilization in PEG with bead size of 3 mm. However, the beads movement or contact between cells and wastewater may be reduced by the carrier of 5 mm, due to larger bead size. Consequently, less H_2 was produced by bacteria immobilization in carrier of 5 mm. From the above finding it may be concluded that the beads of 3 mm offered lesser diffusion resistance compared to the larger beads.

The possibility of re-using of PEG-immobilized cells for H_2 production was studied by periodically replenishing POME (data not shown). The beads were either not washed or washed before adding POME. When the immobilized cell beads were not washed, H_2 yield and production rate production rate decreased gradually and significantly as POME addition was repeated. Compared to the first run, H_2 production decreased by 32% and 61% in the second and third runs, respectively. In comparison, when the immobilized cell beads were washed before adding new POME, the decrease of H_2 production was much less significant. For the latter case, the second run showed 10% decrease compared to the first run and the third run did not show any further decrease compared to the second run.

4. Conclusions

PEG prepolymer served as a good matrix for the entrapment of *C. butyricum* EB6 for H_2 production. The maximum rate of H_2 production with acclimation was 510 mL H_2/L-POME h at an organic loading of 60,000 mg COD/L-POME, compared with 349 mL

H_2/L-POME for unacclimated immobilized cells. The effects of medium concentration (POME) on the H_2 production rate were well explained by Monod-type kinetics. K_P and R_{max,H_2} values for acclimated cells were 20,000 mg COD/L and 490 mL/g of cell/h, respectively. However, for unacclimated immobilized cultures the values were 30,000 mg COD/L and 315 mL/g of cell/h, respectively. The optimum conditions for H_2 production were a bead size of 3.0 mm. SEM micrographs illustrated that the PEG beads were porous structure, which facilitated the transport of substrate and product, ensuring the growth of the cells. This work has shown that the use of PEG-immobilized cells can serve as an excellent alternative for accelerate H_2 production from real wastewater.

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